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Obese father's metabolic state, adiposity, and reproductive capacity indicate son's reproductive health

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Running Title: Father's health predicts son's fertility

Full Title: An obese father's metabolic state, adiposity and reproductive capacity indicate a son's reproductive health

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CAPSULE

Diet and exercise interventions in obese founder males improve the reproductive health of male offspring with a father's metabolic and reproductive status predicting his son's reproductive health.

ABSTRACT

Objective: To determine if dietary and exercise regimes in obese males can provide a novel intervention window for improving the reproductive health of the next generation.

Design: Experimental animal study

Setting: University research facilities

Animal(s): C57BL6 male and female mice

Intervention(s): Mice were fed a control diet (CD, 6% fat) or a high fat diet (HFD, 21% fat) for 9 weeks. After the initial feeding HFD males were allocated to diet and/or exercise interventions for a further 9 weeks. Post intervention males were mated with females fed standard chow (4% fat) before and during pregnancy.

Main Outcome Measures: F1 sperm motility, count, morphology, capacitation, mitochondrial function, and sperm binding and weight of reproductive organs.

Results: Our primary finding was that diet intervention alone in founders improved offspring sperm motility and mitochondrial markers of sperm health (decreased ROS and mitochondrial membrane potential) ultimately improving sperm binding. Sperm binding and capacitation was also improved in F1 males born to combined diet and exercise intervention in founders. Founder sperm parameters and metabolic measures as a response to the diet and/or exercise, (i.e. lipid/glucose homeostasis, sperm count and morphology) correlated with offspring's sperm function independent of founder treatment. This implicates paternal metabolic and reproductive status in predicting male offspring's reproductive function.

Conclusion: This is the first study to show that improvements to both metabolic (lipids, glucose and insulin sensitivity) and reproductive function (sperm motility and morphology) in obese fathers via diet and exercise interventions can improve subsequent reproductive health in offspring.

KEYWORDS:

- Transgenerational
- Sperm
- Obesity
- Diet and/or exercise
- Interventions

INTRODUCTION

Peri-conception paternal health has been shown to influence the health of subsequent children. For example paternal smoking, age and occupational chemical exposure are associated with an increased risk of impaired child health (El-Helaly et al., 2011; Lee et al., 2009; Van Balkom et al., 2012). Recent epidemiological studies demonstrate that paternal nutritional status and obesity are correlated with altered child health outcomes (Danielzik et al., 2002; Li et al., 2009). However, human studies are confounded by the common environmental exposures shared by both father and child.

Recent rodent models of paternal obesity have demonstrated that male offspring reproductive function was impaired, as evident by increased sperm intracellular reactive oxygen species (ROS), reduced sperm motility and reduced sperm binding (Fullston et al., 2012). Interestingly the same impairments persisted into second generation males (Fullston et al., 2012). The relevance of these findings are highlighted in western societies, as currently 70% of reproductive aged men are overweight or obese (2013) suggesting likely changes to offspring reproductive health. Therefore, lifestyle changes in obese men may provide an unappreciated novel intervention window to maximise the reproductive function of the next generation.

Diet and exercise interventions in obese males have recently been shown to improve sperm parameters. Both gastric bypass surgery and weight loss through scheduled diet and exercise programs have resulted in improvements to sex hormone profiles, sexual function, total sperm count and morphology in men who lost the greatest amount of weight (Hakonsen et al., 2011; Reis et al., 2012). The extent to which either the metabolic profile or adiposity enacted these outcomes remains to be investigated. In a mouse model of male obesity diet and exercise interventions normalised levels of sperm ROS, DNA damage and sperm binding (Palmer et al., 2012), parameters which subsequently improved embryo development and fetal size (Mcpherson et al., 2013).

Altogether the evidence highlights the potential of weight loss strategies to restore sperm function of obese males in both rodent models and humans. To date no studies have determined whether weight loss and improved metabolic status in obese males can reverse the adverse reproductive effects in their male offspring. We therefore used our mouse model of male obesity to assess the hypothesis that a reduction in adiposity and/or an improvement to metabolic health via diet and exercise in obese fathers will improve reproductive health in their male offspring.

MATERIALS AND METHODS

Founder Animals and Diet

Five week old male C57BL6 mice (n=40) were randomly assigned to one of two diets for an initial period of 9 weeks: 1) control diet (CD; n=8) (SF04-057; Specialty Feeds, Perth, Australia); or 2) a high fat diet (HFD; n=32) high in fat and nutrient matched (SF00-219; Specialty Feeds, Perth, Australia) (Supplementary Table 1). The HFD used in the study has been previously shown to increase adiposity and impair sperm function compared with the nutritionally matched CD (Bakos et al., 2011; Brake et al., 2006; Mitchell et al., 2011; Palmer et al., 2011). After the initial feeding period, males allocated to the HFD were randomly allocated to one of the following interventions for a further 9 weeks: 1) continuation of a HFD (HH) (n=8); 2) change to a CD (HC) (n=8); 3) continuation of a HFD with exercise (HE) (n=8); 4) change to a CD with exercise (HCE) (n=8). Mice allocated to the CD during the initial feeding period continued to be fed a CD during the intervention period as a baseline control (CC). The previously described swimming intervention regime simulates light exercise (Mcpherson et al., 2013; Palmer et al., 2012) and demonstrated not to cause additional stress whereby the mice are given gradual acclimatisation to the full exercise program over two weeks (Palmer et al., 2012). Male body weights were recorded weekly both pre and post intervention. Metabolic status of founder males was obtained via fasted glucose tolerance test (GTT, expressed as area under curve (AUC)), fed insulin tolerance test (ITT, expressed as area above curve (AAC)) at 7 and 8 weeks respectively as per (Palmer et al., 2012) and fasting post mortem plasma measures of cholesterol, free fatty acids (FFA), glucose and triglycerides as previously described in (Gatford et al., 2009). Animals were individually housed in a 12:12 h dark light cycle for the entire study, fed *ad libitum* and given free access to water. The use and care of all animals used in the study was approved by the Animal Ethics Committee of The University of Adelaide.

Generation/Sampling of F1 Males

At 7 weeks post intervention (21 weeks of age) founder males were paired with 2 normal weight 8-10 week C57BL6 females for a maximum period of 8 nights. Female mice were housed with founder males during the dark cycle only and separated and maintained standard chow during the light cycle. Successful mating was assessed the following morning by the presence of a vaginal plug. HH founders had a reduced number of successful matting's compared with HCE and CC founders ($p<0.05$, data not shown). After successful mating female mice were group housed until day 15 of pregnancy and then individually housed until offspring were weaned. Females were maintained on standard chow during pregnancy and post birth. Females were allowed to pup and at weaning 1 male from each litter was randomly sampled for reproductive health measurements. For the CC group 8 F1 males were sampled from 8 litters representating 6 founders. For the HH group 10 F1 males were sampled from 10 litters representating 7 founders. For the HC treatment 10 F1 males were sampled from 10 litters representating 7 founders. For the HE treatment 10 F1 males were sample from 10 litters representating 7 founders and for the HCE treatment 8 F1 males were sampled from 8 litters representating 6 founders. F1 males were group housed independently of founder treatment and maintained on standard chow.

Sperm Collection, Count, Motility and Morphology Analysis

Sperm were collected immediately post mortem from the cauda epididymis and ductus deferens and expressed into 1 ml of G-IVF medium (Vitrolife, Gothenberg, Sweden) and incubated for at least 10 min in 6% CO_2 and 5% O_2 at 37°C (Bakos et al., 2011). Sperm count, motility and morphology were assessed blinded in accordance with WHO guidelines (Who & World Health Organisation, 2010), with at least 200 sperm from each sample measured. Sperm motility was assessed by classifying sperm as either progressive motile, non progressive motile or immotile. Motility was expressed as a percentage for both progressive motile and total motility (combination of both progressive motile and non progressive motile sperm). Sperm morphology was assessed on samples fixed with methanol:acetone

(3:1) and stained with haematoxylin and eosin. Sperm morphology of individual sperm were scored as normal, tail defect or head defect as per (Palmer et al., 2012) and expressed as a percentage of each form.

F1 Male Sperm Binding

The numbers of sperm bound to the zona pellucida of an MII oocyte were assessed as described in (2011). At least 10 oocytes were analysed per sperm sample.

F1 Male Sperm Capacitation and Acrosome Reaction

Capacitation and acrosome reaction were measured using *Arachis Hypogaea* (peanut) agglutinin (Lectin PNA; Molecular Probes, Eugene, USA) as previously described (Baker et al., 2004; Bakos et al., 2011). A minimum of 200 sperm were counted per sample. The proportion of non-capacitated, capacitated and acrosome reacted sperm were expressed as a percentage.

F1 Male Sperm Mitochondrial ROS assay & Vitality Measure

The intracellular generation of mitochondrial ROS was determined using MitoSox Red (MSR; Molecular Probes, Eugene, USA) and SytoxGreen (Molecular Probes) as previously described (Koppers et al., 2010). Both negative (sperm incubated only in SytoxGreen) and positive (sperm incubated in 1500 μM of H_2O_2) controls were conducted. MSR and SytoxGreen fluorescence was measured on a FACSCanto flow cytometer (BD Bioscience, North Ryde, Australia). Non-specific sperm events were gated out and 20,000 cells were examined per sample. MSR results were expressed as percent of live sperm positive for MSR. Vitality was measured as the percentage of sperm that did not display SytoxGreen fluorescence.

F1 Male Sperm Mitochondrial Membrane Potential (MMP) (JC-1)

Sperm mitochondrial membrane potential was determined by using the ratiometric dye JC-1 (Molecular Probes, Eugene, USA) as previously described (2010). A negative control was also included whereby sperm were incubated in 10 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate membrane potential before incubation in JC-1. JC-1 and PI fluorescence were measured on a FACSCanto flow cytometer. Non-specific sperm events were gated out and 20,000 cells were examined per sample. Results were expressed as the percent of live sperm positive for a high JC-1 reading.

Adiposity and Reproductive Organ Measurement

Founder males at pre intervention week 9 (14 weeks of age) and post intervention week 8 (22 weeks of age) and 10 weeks of age for F1 male offspring, total adiposity was measured by a dual-emission X-ray absorptiometry machine (DEXA) (Piximus, Ge Lunar, Wisconsin, USA) as previously described (Nagy & Clair, 2000). At intervention week 9 (23 weeks of age) for founder males and 10 weeks of age for F1 males gonadal fat, testes and seminal vesicles were dissected and weighed post mortem. All dissections and weighing were performed blinded to treatment group and performed by the same investigator.

Testosterone analysis

At intervention week 9 for founder males (23 weeks of age) and 10 weeks of age for F1 males, serum testosterone was measured by a stable-isotope dilution liquid chromatography coupled with tandem mass spectrometry as previously described (Bakos et al., 2011; Mcnamara et al., 2010).

Statistics

All data were expressed as mean ± SEM and checked for normality using a Kolmogorov-Smirnov test and equal variance using a Levene's test. All statistical analysis was performed in SPSS (SPSS

Version 18, SPSS Inc., Chicago, USA) with an observed power of ≥80%. A p value <0.05 was considered to be significant.

Founder Measures

Founder reproductive and metabolic changes were determined by a univariate general linear model. Cohort of animals and replicate were fitted as covariates

F1 Male Measures

To compare F1 male offspring sperm parameters, reproductive organs and testosterone levels across the 5 treatments, linear mixed effect models were fitted. In the model father ID was included as a random effect to adjust for dependence in results between offspring from the same father and litter size as a dependent variable to compared litter size variations between and within the 5 treatments. Correlations between F1 male offspring reproductive health and founder metabolic and reproductive health were determined by multiple regression analysis and corrected for multiple observations.

RESULTS

Effect of Diet and Exercise on Founder Metabolism and Sperm Parameters

Founders fed a HFD during the pre-intervention period had a 20.4% increase in body weight compared with founders fed a control diet (31.6 ± 0.69 vs. 26.3 ± 0.47, $p<0.05$). Post intervention CC, HH and HE founders continued to gain weight (13.6%, 15.8% and 6.4% respectively, Table 1), while interventions involving diet (HC and HCE) reduced founder body weight (-4.4% and -11.0% respectively, Table 1). Founder male post-intervention metabolic and reproductive phenotypes recapitulated previous findings (Mcpherson et al., 2013; Palmer et al., 2012). Diet interventions (HC and HCE) reduced adiposity (total and gonadal), serum cholesterol and glucose tolerance compared with HH founders ($p<0.05$, Table 1). Founders who received exercise intervention alone (HE) maintained their increased serum cholesterol and insulin resistance (Table 1) while sustaining their pre intervention level of adiposity such that is was still increased compared with CC founders ($p<0.05$, Table 1), however reduced compared with HH founders ($p<0.05$, Table 1). Interventions including exercise (HE and HCE) improved glucose clearance compared with HH founders ($p<0.05$, Table 1) with HE founders also reducing fasting glucose ($p<0.05$, Table 1). All interventions reduced the number of sperm with tail defects compared with HH founders ($p<0.05$, Table 1) while exercise interventions in founders (HE and HCE) additionally restored sperm motility ($p<0.05$, Table 1). Diet intervention alone also increased serum testosterone levels compared with HH founders ($p<0.05$, Table 1) restoring it to levels of CC founders.

Effect of Founder Diet and Exercise on F1 Conventional Sperm Parameters

HH founders produced F1 males with reduced progressively motile sperm compared with males born to CC founders ($p<0.05$, Table 2). Diet intervention alone in founders (HC) increased the percentage of progressively motile sperm in F1 males compared with F1 males born by HH founders ($p<0.05$, Table 2), restoring to level that of F1 males born by CC founders while exercise interventions in founders with (HCE) or without dietary intervention (HE) did not improve percentage of progressively motile sperm in

F1 males (Table 2). There was no change in the total proportion of motile sperm or proportion of immotile sperm from F1 males born from any founder treatment group (Table 2). Founder treatment group had no effect on the proportion of normal sperm morphology in F1 males (Table 2), although the proportion of head and tail defects in sperm were altered among groups ($p<0.05$, Table 2). HH founders produced F1 males with reduced sperm counts compared with F1 males born by CC founders ($p<0.05$, Table 2). There were no significant improvements to F1 sperm count from diet and/or exercise interventions in founders compared with F1 male born by HH founders (Table 2).

Effect of Founder Diet and Exercise on F1 Sperm Capacitation and Oocyte Binding

HH founders did not produce F1 males with any difference in sperm capacitation compared with CC founders (CC), concordant with previous reports using shorter feeding periods (Fullston et al., 2012). However, all diet and/or exercise interventions in founders (HC, HE and HCE) increased the percentage of capacitated F1 sperm compared with F1 males born to HH or CC founders ($p<0.05$, Table 2), conversely reducing non capacitated and acrosome reacted sperm (Table 2).

HH founders produced F1 males with reduced sperm binding compared with F1 males born to CC founders ($p<0.05$, Table 2). Diet intervention with (HCE) or without (HC) exercise in founders increased sperm binding compared with F1 males born from HH founders ($p<0.05$, Table 2) with F1 males born to combined diet and exercise (HCE) intervention in founders exceeding numbers of F1 males born by CC founders ($p<0.05$, Table 2). In contrast, exercise alone intervention (HE) in founders did not improve sperm binding in F1 males compared to born to HH founders ($p>0.05$, Table 2).

Effect of Founder Diet and Exercise on F1 Sperm Mitochondrial Parameters

There was no effect of HH founders on F1 levels of sperm ROS compared with F1 males born to CC founders, which was previously reported in shorter feeding periods (Fullston et al., 2012). Diet intervention alone in founders (HC) reduced MSR positive sperm in F1 males compared with F1 males born from CC, HE and HCE founders ($p<0.05$, Table 2). Once more a founder intervention limited solely to diet (HC) reduced the proportion of F1 sperm positive for high JC1 compared with F1 males born to HH, and HCE founders ($p<0.05$, Table 2), while exercise intervention alone (HE) had no effect (Table 2).

Effect of Founder Diet and Exercise on F1 Male Reproductive Body Composition and Serum Testosterone

There was no effect of founder treatment on F1 male body composition as assessed by total body weight, total adiposity, gonadal adiposity, seminal vesicle weights, testes weights or serum testosterone concentration ($p>0.05$, Table 3). However, it must be noted that the similarities in testosterone results in offspring to that of their fathers was further confirmed by founder serum testosterone positively correlating with offspring serum testosterone (0.693, $p<0.001$, Table 4).

Correlations of Founder Adiposity and Metabolic status with F1 Reproductive Function

We have previously shown that founder metabolites independent to treatment group correlated with founder male sperm function, embryo development and early fetal health markers (Mcpherson et al., 2013; Palmer et al., 2012) with perturbed sperm function seen in F1 males produced by founders with increased adiposity and serum cholesterols (Fullston et al., 2012). Given the variations of founder metabolites within treatments, the varied improvements to F1 male sperm parameters across treatments and the systematic changes to whole body physiology from obesity impacting on spermatogenesis (i.e. hyperglycaemia) we further examined if measures of founder metabolic health correlated with F1 male reproductive measures. We hypothesised that the differing levels of adiposity and metabolic health in founders both between and within treatment groups could help explain their son's reproductive measures. Correlations highlighted that F1 male reproductive health (sperm motility, sperm binding, sperm count, total body weight, testes weights, seminal vesicle weights, gonadal adiposity and testosterone levels) is sensitive to founder gonadal adiposity, serum FFA/triglyceride/cholesterol concentrations, glucose tolerance and insulin sensitivity ($p<0.05$, Table 4).

Correlations of Founder Reproductive Function with F1 Reproductive Function

Commonly observed sub-fertility phenotypes seen in obese men, such as reduced sperm motility, count and morphology have been associated with chromatin and epigenetic modifications in sperm (Hammoud et al., 2011; Iranpour et al., 2000). We hypothesised that sperm parameters in founders may indicate epigenetic modifications in sperm which might form the basis for their offspring inheriting similar sub fertility phenotypes. Correlations revealed that an F1 male's reproductive measures are also sensitive to a founder male's reproductive health (Table 4). For example founder sperm levels of normal morphology correlated positively with F1 testes weights ($p=0.04$) and negatively with F1 sperm positive for MSR ($p<0.01$, Table 4).

DISCUSSION

To date the end points for assessing the reversibility of paternal obesity and associated co-morbidities have been limited to hormone profiles (Bastounis et al., 1998; Hammoud et al., 2009; Strain et al., 1988), sperm function (Hakonsen et al., 2011; Reis et al., 2012) and extended as far as early embryo development and quality in rodent models of paternal obesity (Mcpherson et al., 2013). This is the first study to further determine if the altered reproductive phenotypes in male offspring that results from an obese father can be improved by lifestyle interventions. We demonstrate that diet and/or exercise interventions in obese fathers can improve the reproductive health of male offspring in a mouse model, demonstrating that adiposity, metabolic and reproductive status of fathers influences their son's reproductive health. If translatable to the human this data suggests a potential novel pre-conception intervention window for obese fathers to improve their male offspring's reproductive health via lifestyle interventions.

Sperm motility and concentration are standard parameters for assessing male fertility (2010). While additional measures of sperm capacitation and binding (Franken & Oehninger, 2006; Liu et al., 2004) can be indications of sperm function, due to the essential nature of these processes for penetration into the oocyte during fertilisation (Johnson & Everitt, 2000). Further, sperm ROS concentrations are a further marker of sperm health with increased levels in human sperm correlating with reduced fertilisation, impaired embryonic development and pregnancy loss (Dada et al., 2010; Gharagozloo & Aitken, 2011; Tunc et al., 2010; Zribi et al., 2011).

In a mouse model, it has been previously reported that in addition to effects on founder male sperm, male offspring born from HFD fathers also had reduced sperm motility, reduced sperm binding and increased levels of ROS (Fullston et al., 2012). This study recapitulated the reduced sperm motility and sperm binding of male offspring born from HFD (HH) fathers, although we did not report similar increases in levels of ROS. This could be due to a number of reasons including duration of founder exposure to diet (10 weeks vs. 18 weeks) or different ROS detection method (DCFDA vs. MitoSox Red) which may explain the differences.

Diet intervention alone in obese founders restored adiposity, glucose homeostasis and cholesterol levels similar to human studies (Klop et al., 2013). These founders produced male offspring with improvements to sperm mitochondrial health (ROS and membrane potential) which were sometimes below levels of controls (CC), increased sperm motility, binding and capacitation compared with F1 males born to HH founders. This suggests that simple caloric restriction in obese males restores adiposity as well as glucose/lipid metabolism and can improve subsequent male offspring sperm function, which would potentially increase embryo development and pregnancy rates for this F1 generation. Combined diet and exercise interventions (HCE) in founders also increased the functional measure of sperm binding in their male offspring likely resulting from increased sperm capacitation and improvements to sperm motility which again suggests likely improvements to fertilisation.

Interestingly, the continuation of a HFD with an exercise intervention in founders (HE) showed the least improvements to offspring sperm function, with only slight improvements to sperm capacitation compared with F1 males from HH founders. Founder males in this group, maintained their pre intervention level of adiposity compared with founders undergoing diet interventions (HC or HCE) and controls (CC), indicating that the exercise regime saw the similar amount of calories expended as was ingested. HE founders also maintained their increased serum cholesterols likely resulting from the lipid dense diet consumed. This is a similar phenotype to that observed in exercise intervention alone in obese humans which have shown that exercise alone does not reduce adiposity levels (Dwyer-

Lindgren et al., 2013). This implies that improvements to these aspects of sperm function in male offspring maybe related to the adipose state and cholesterol levels of their father.

Increased scrotal heat due to increased adiposity in humans is associated with reduced sperm motility, morphology, increased sperm DNA damage and increased sperm oxidative stress (Paul et al., 2008a; Paul et al., 2008b; Shiraishi et al., 2010), parameters which have been independently linked with epigenetic changes to sperm (Hammoud et al., 2011; Iranpour et al., 2000; Tunc & Tremellen, 2009). Therefore as our males fed a HFD with (HE) maintain gonadal adiposity, increased scrotal heat could potentially underpin the transmission of altered offspring health seen. However, it should be noted that offspring from males fed a HFD with exercise (HE) interventions did have some improvements in sperm function (motility and morphology), suggesting that heat may not be the sole mechanism for the adverse changes to offspring sperm function seen in the current study.

The precise mechanisms that are responsible for the transmission of altered offspring health due to paternal obesity remain unidentified. Epigenetic, molecular and functional changes within the sperm either through changes to epigenetic marks to sperm DNA and sperm chromatin structure are clearly implicated. Direct insults to founder male sperm DNA such as irradiation induced sperm DNA damage, or gamete and somatic cell DNA damage induced by impaired intra-uterine environments from mothers (Adiga et al., 2010) leads to impaired reproductive function (Fullston et al., 2012) and changes to body composition in first generation offspring (Dunn & Bale, 2011). This links the molecular composition of father's sperm at the time of conception to the health of the next generation. There is emerging evidence that increased adiposity in males impacts the epigenetic status of their sperm. Correlations with markers of founder metabolic health and offspring sperm function determined that founder gonadal adiposity negatively correlated with offspring's sperm count and positively with the proportion of non progressive motile sperm independent of treatment group. Global measures of methylation in testes and elongating spermatids showed that DNA from cells were hypomethylated in obese male mice (Fullston et al., 2013). Obesity additionally alters the methylation status of DNA in other tissues (Barres & Zierath, 2011). Whether the changes to reproductive function in our male offspring results from a global alteration to de novo methylation or site specific methylation at paternally imprinting gene loci in the obese father's sperm, remains to be determined. Sperm also harbour a vast array of small non-coding RNAs which are thought to be important for early embryo and fetal development (Liu et al., 2012) and have been previously shown to be altered in sperm of obese rodents (Fullston et al., 2013). Interestingly, changes to circulating serum microRNAs caused from obesity were restored through weight loss (Ortega et al., 2013) providing evidence that interventions to health can change the microRNA content of specific tissues. Whether the diet interventions (HC and HCE) which induced weight loss could also restore the microRNA content of testes and sperm and identify a potential part of the mechanism that might improve reproductive function in offspring remains to be determined.

We have previously demonstrated that metabolic markers in fathers including plasma lipids, glucose homeostasis and insulin sensitivity can impact on both their sperm function and F1 embryo development independently of adiposity (Mcpherson et al., 2013; Palmer et al., 2012). In this study, founder plasma measures of glucose, insulin and fatty acid metabolism which showed the biggest restoration in diet interventions displayed the strongest correlations with male offspring sperm function and body composition including total body weight, testes and seminal vesicle weights. Increased circulating levels of serum lipids in men, have been associated with increased ROS in sperm (Koppers et al., 2010) and changes to the global methylation of sperm DNA (Tunc & Tremellen, 2009). Additionally fasting plasma insulin concentrations in fathers can predict umbilical cord insulin levels and therefore offspring fetal size (Shields et al., 2006). Together these data suggest that pre-conception paternal metabolic status may alter the epigenetic signature of sperm, programming offspring phenotypes thereby providing part explanations for our correlations between markers of paternal metabolic state with their son's reproductive phenotypes.

Recently it has been demonstrated that the proteomic, mRNA and microRNA content of sperm/testes are altered in obese rodents (Daxinger & Whitelaw, 2012; Fullston et al., 2013; Ghanayem et al., 2010; Kriegel et al., 2009; Palmer et al., 2011; Youngson & Whitelaw, 2011). In agreement with our previous study (Palmer et al., 2012), we report here that diet and exercise interventions in founders improves their sperm function, suggesting lifestyle interventions may at least in part restore the micro-molecular environment of testes and/or epididymis thereby restoring the molecular makeup of sperm. The concept that diet and exercise interventions restore the micro-environments of reproductive organs and therefore restores the molecular determinants responsible for programming offspring health in sperm is supported by the associations of founder sperm parameters of morphology, count and motility with F1 reproductive parameters of sperm morphology, capacitation, testes weights and serum testosterone levels.

The model described of feeding a HFD to induce obesity mimics some aspects of human obesity including increased adiposity serum cholesterol and altered glucose and insulin homeostasis (Klop et al., 2013), with diet and exercise interventions used previously shown to restore sperm function and DNA integrity (Palmer et al., 2012), similar to those reported improvements to sperm parameters found in diet and exercise interventions in human studies (Hakonsen et al., 2011). Due to the similar metabolic and sperm function phenotypes of both rodent models of obesity and obese humans with diet and exercise interventions suggests that the changes and or improvements to the testicular microenvironment is likely similar and therefore the molecular changes proposed to founder sperm to induce F1 male offspring phenotypes could act through similar pathways and be translatable to humans. However the differences in sperm molecular makeup between both mouse and humans still need to be noted. For example it has been proposed that human sperm are much more sensitive to environmental perturbations than mouse sperm due to their higher levels of histone retention (~15% human (Gatewood et al., 1987) compared with ~1% in the mouse (Balhorn et al., 1977)) which are capable of normal histone modifications (Farthing et al., 2008) and are retained at loci that contain genes important for early embryogenesis (Farthing et al., 2008). The similarities between both human and rodent models of male obesity suggest that these results would likely be translatable to humans; however confirmation studies in a human cohort would still be warranted.

This report shows that impaired offspring reproductive health resulting from paternal obesity can be improved through weight loss and restoration of metabolic health in fathers via diet interventions. Additionally paternal markers of adiposity, metabolism and reproductive function may also indicate their son's reproductive function, thus potentially highlighting a novel intervention window for improving reproductive health outcomes in the next generation. The direct sperm molecular mechanism responsible for this improvement transmitted via the father and if this can be replicated in humans to improve reproductive outcomes in the next generation, warrants further investigation.

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Table 1: Effect of Diet and Exercise on Founder Adiposity, Serum Metabolites and Reproductive Measures

	Diet/Intervention				
	CC	**HH**	**HC**	**HE**	**HCE**
Pre intervention					
Body weight (g)	26.3 ± 0.47[a]	31.5 ± 0.82[b]	31.8 ± 1.5[b]	30.8 ± 1.3[b]	32.6 ± 1.9[b]
Total adiposity (% of body weight)	14.6 ± 0.92[a]	24.7 ± 1.7[b]	24.7 ± 2.0[b]	24.5 ± 1.7[b]	26.0 ± 1.6[b]
Post intervention					
Final body weight (g)	29.9 ± 0.58[a]	36.3 ± 1.14[b]	30.4 ± 1.6[a]	32.8 ± 1.2[a]	29.2 ± 1.1[a]
Total adiposity (% of body weight)	16.4 ± 0.88[a]	28.4 ± 2.00[b]	18.1 ± 2.15[a]	24.0 ± 1.19[c]	16.8 ± 1.18[a]
Gonadal Adiposity (% total body weight)	2.46 ± 0.2[a]	4.82 ± 0.28[b]	2.82 ± 0.27[a]	4.06 ± 0.27[c]	2.77 ± 0.28[a]
Glucose (mmol/L)	10.0 ± 0.51[ab]	10.6 ± 0.42[a]	9.8 ± 0.47[ab]	9.4 ± 0.48[b]	9.7 ± 0.44[ab]
Cholesterol (mmol/L)	3.24 ± 0.36[ac]	4.11 ± 0.30[b]	3.17 ± 0.34[ac]	3.82 ± 0.34[ab]	2.39 ± 0.36[c]
FFA (mmol/L)	0.69 ± 0.05[a]	0.64 ± 0.04[ab]	0.61 ± 0.04[ab]	0.65 ± 0.04[ab]	0.55 ± 0.05[b]
Triglycerides (mmol/L)	0.77 ± 0.06	0.83 ± 0.05	0.78 ± 0.06	0.83 ± 0.06	0.80 ± 0.06
Glucose (AUC)	1797 ± 122[a]	2111 ± 100[b]	1814 ± 123[a]	1695 ± 113[a]	1787 ± 103[a]
Insulin (AAC)	167 ± 13.4[a]	121 ± 10.1[b]	135 ± 12.2[ab]	75 ± 12.4[c]	111 ± 12.2[b]
Reproductive Measures					
Testosterone (ng/L)	0.36 ± 0.14[a]	0.10 ± 0.03[b]	0.38 ± 0.15[a]	0.28 ± 0.11[ab]	0.17 ± 0.04[ab]
Testes weights (g)	0.16 ± 0.05	0.17 ± 0.04	0.16 ± 0.04	0.16 ± 0.03	0.16 ± 0.05
Seminal vesicle weights (g)	0.37 ± 0.03	0.39 ± 0.03	0.36 ± 0.02	0.34 ± 0.03	0.36 ± 0.03
Sperm count (10^6)	21.7 ± 3.1	20.0 ± 3.3	16.9 ± 3.1	22.2 ± 2.9	22.1 ± 3.3
Progressive sperm motility (%)	31.9 ± 5.7	26.2 ± 6.1	28.9 ± 5.7	38.6 ± 5.3	39.8 ± 6.1
Total sperm motility (%)	71.3 ± 3.9[a]	58.3 ± 4.2[b]	66.6 ± 3.9[ab]	74.5 ± 3.6[a]	72.9 ± 4.2[a]
Sperm normal forms (%)	56.9 ± 1.8[a]	55.9 ± 3.1[a]	59.3 ± 1.9[ab]	64.1 ± 2.2[b]	58.7 ± 3.7[ab]
Sperm with tail defects (%)	30.8 ± 2.1[a]	36.4 ± 3.0[b]	29.9 ± 1.3[a]	29.7 ± 2.7[a]	30.7 ± 2.5[a]
Sperm with head defects (%)	12.2 ± 2.4[a]	8.6 ± 1.9[ab]	10.6 ± 1.1[ab]	6.1 ± 0.7[b]	10.51 ± 0.7[ab]

Data is representative of 8 founder males per treatment group with sperm morphology representative of 4 founder males per treatment group and expressed per male. Testes weights were a combined weight of both left and right testis. Data was analysed by a GLM with cohort and replicated fitted as covariates. Different letters denote significance at $p<0.05$.

Table 2: Effect of Founder Diet and Exercise on F1 Sperm Function

F1 Sperm Measures	Founder Diet/Intervention				
	CC	HH	HC	HE	HCE
Conventional Sperm Parameters					
Progressive sperm motility (%)	32.2 ± 6.4^{a}	18.1 ± 5.1^{b}	31.2 ± 6.0^{a}	22.1 ± 6.0^{ab}	23.0 ± 5.6^{ab}
Total sperm motility (%)	53.1 ± 5.2	45.9 ± 4.3	49.2 ± 4.9	45.6 ± 4.6	46.9 ± 4.4
Immotile sperm (%)	48.1 ± 4.7	54.5 ± 3.7	48.6 ± 4.7	56.7 ± 4.3	54.6 ± 4.1
Sperm normal morphology (%)	56.7 ± 4.3	50.6 ± 3.6	50.7 ± 4.1	51.8 ± 4.0	49.1 ± 3.7
Sperm head defect (%)	10.8 ± 1.5^{ab}	11.6 ± 1.3^{ab}	14.8 ± 1.5^{a}	9.4 ± 1.4^{b}	10.4 ± 1.3^{b}
Sperm tail defect (%)	32.3 ± 3.4^{a}	37.7 ± 2.8^{ab}	34.4 ± 3.3^{ab}	38.6 ± 3.2^{ab}	40.4 ± 2.9^{b}
Sperm count (10^6)	12.3 ± 1.1^{a}	10.0 ± 0.9^{b}	11.1 ± 1.2^{ab}	8.2 ± 1.0^{b}	10.2 ± 1.1^{ab}
Sperm Capacitation and Oocyte binding					
Sperm non capacitated (%)	2.36 ± 0.38^{ab}	3.04 ± 0.30^{a}	2.05 ± 0.34^{b}	1.54 ± 0.31^{b}	1.27 ± 0.33^{b}
Sperm capacitated (%)	94.3 ± 0.6^{a}	94.6 ± 0.5^{a}	96.5 ± 0.6^{b}	96.2 ± 0.5^{b}	96.6 ± 0.6^{b}
Sperm acrosome reacted (%)	3.33 ± 0.39^{a}	2.35 ± 0.25^{ac}	1.42 ± 0.28^{bc}	2.24 ± 0.26^{c}	2.12 ± 0.64^{c}
Sperm bound to MII oocyte	48.0 ± 1.2^{a}	37.6 ± 1.3^{b}	42.9 ± 1.5^{c}	40.7 ± 1.2^{bc}	52.9 ± 1.5^{d}
Sperm Mitochondrial Function					
Sperm positive for MSR (%)	61.3 ± 4.3^{a}	57.1 ± 3.6^{ab}	49.6 ± 4.0^{b}	60.5 ± 4.0^{a}	60.8 ± 3.6^{a}
Vitality (%)	58.8 ± 4.9	60.7 ± 4.1	65.5 ± 4.6	63.0 ± 4.3	56.9 ± 4.1
Sperm positive for high JC1 (%)	52.4 ± 4.1^{ab}	55.6 ± 3.4^{a}	48.1 ± 3.8^{b}	51.3 ± 3.8^{ab}	60. 3 ± 3.3^{a}

8 F1 CC and HCE males and 10 HH, HC and HE males were analysed and expressed per animal representative of 6 CC and HCE founders and 7 HH, HC and HE founders. MSR H_2O_2 positive control was 73.4% and MSR negative control was 4.5%. CCCP negative control for high JC1 was 17.1% and PI only negative control for high JC1 was 2.7%. Data was analysed by a linear mixed effects model with father ID added as a random effect and litter size as a fixed variable. Different letters denote significance at $p<0.05$.

Table 3: Effect of Founder Diet and Exercise on F1 Male Reproductive Organs and Testosterone

	Founder Diet/Intervention				
F1 weight	**CC**	**HH**	**HC**	**HE**	**HCE**
Total body weight (g)	22.0 ± 0.3	22.1 ± 0.3	21.8 ± 0.3	21.3 ± 0.3	22.7 ± 0.3
Total adiposity (g) [#]	1.65 ± 0.06	1.57 ± 0.07	1.51 ± 0.07	1.51 ± 0.06	1.53 ± 0.06
Gonadal adiposity (g)[#]	0.146 ± 0.016	0.152 ± 0.014	0.158 ± 0.016	0.168 ± 0.015	0.145 ± 0.016
Testes (g)[#]	0.145 ± 0.003	0.155 ± 0.003	0.154 ± 0.003	0.149 ± 0.003	0.153 ± 0.003
Seminal Vesicles (g)[#]	0.149 ± 0.010	0.155 ± 0.009	0.42 ± 0.011	0.150 ± 0.010	0.165 ± 0.010
Testosterone (ng/L)	0.167 ± 0.061	0.109 ± 0.054	0.166 ± 0.066	0.112 ± 0.073	0.114 ± 0.057

8 F1 CC and HCE males and 10 F1 HH, HC and HE males were analysed and expressed per male representative of 6 CC and HCE founders and 7 HH, HC and HE founders. Testes weights were a combined weight of both left and right testis. [#]No significant differences in total or gonadal adiposity, testes or seminal vesicle weights when expressed as percentage of total body weight. Data was analysed by a linear mixed effects model with father ID added as a random effect and litter size as a fixed variable.

Table 4: Founder Metabolic and Reproductive Health Correlate with F1 Reproductive Measures

F1 Measure	Founder Measure	Correlation Coefficient	P Value
Sperm Function			
Progressive sperm motility (%)	FFA (mmol/L)	-0.557	***0.024***
Non progressive sperm motility (%)	Gonadal adiposity (%)	0.489	***0.045***
Sperm head defect (%)	Sperm count (10^6)	-0.539	***0.029***
Sperm positive for MSR (%)	Progressive sperm motility (%)	-0.570	***0.027***
	Total motile sperm (%)	-0.829	***<0.001***
	Sperm normal morphology (%)	-0.664	***0.009***
	Sperm head defect (%)	0.474	0.060
Sperm bound to MII oocyte	FFA (mmol/L)	-0.529	***0.038***
	Glucose (mmol/L)	-0.482	0.056
	Glucose (AUC)	-0.551	***0.032***
	Seminal vesicle weights (g)	0.479	0.058
Sperm non capacitated (%)	Progressive sperm motility (%)	-0.565	***0.028***
	Total motile sperm (%)	-0.820	***<0.001***
	Sperm normal morphology (%)	-0.475	0.060
Sperm capacitated (%)	Progressive sperm motility (%)	0.572	***0.026***
	Total motile sperm (%)	0.621	***0.016***
	Sperm count (10^6)	-0.574	***0.026***
Sperm acrosome reacted (%)	Sperm count (10^6)	0.693	***0.006***
Sperm count (10^6)	FFA (mmol/L)	-0.503	***0.040***
	Triglycerides (mmol/L)	-0.440	0.060
	Gonadal adiposity (%)	-0.468	0.050
	Progressive sperm motility (%)	0.808	***<0.001***
	Testes weights (g)	0.644	***0.009***
Reproductive Organs and Testosterone			
Total body weight (g)	Glucose (mmol/L)	-0.512	***0.025***
	Glucose (AUC)	-0.487	***0.033***
	Total motile sperm (%)	-0.433	0.050
Testes (%)	Glucose (mmol/L)	0.466	***0.040***
	Glucose (AUC)	0.521	***0.023***
	Progressive sperm motility (%)	0.607	***0.008***
	Sperm normal morphology (%)	0.623	***0.040***
Seminal vesicles (g)	Glucose (mmol/L)	-0.430	***0.046***
	Glucose (AUC)	-0.443	***0.049***
	Seminal vesicles (g)	0.478	***0.036***
Gonadal adiposity (%)	FFA (mmol/L)	0.487	***0.033***
	Cholesterol (mmol/L)	0.486	***0.040***
Testosterone (ng/L)	Sperm normal morphology (%)	-0.606	***0.024***
	Sperm tail defect (%)	0.517	0.052
	Testosterone (ng/L)	0.693	***<0.001***

Correlations were determined by multiple regression analysis and corrected for multiple observations.

Supplementary Table 1: Composition of Animal Diets

Ingredients	CD (SF04-057) Control Diet	HFD (SF00-219) Harlan Teklad TD88137 Equival
Sucrose (g/100g)	34.1	34.1
Casein (Acid) (g/100g)	19.5	19.5
Canola Oil (g/100g)	6.0	-
Clarified Butter (g/100g)	-	21.0
Cellulose (g/100g)	5.0	5.0
Wheat starch (g/100g)	30.5	15.5
Minerals (g/100g)	4.9	4.9
Digestible energy (MJ/kg)	16.1	19.4
Digestible energy from lipids (%)	21.0	40.0
Digestible energy form protein (%)	14.0	17.0
Digestible energy from carbohydrates (%)	65.0	43.0

CD = Control diet (6% fat) and HFD = high fat diet (21% fat).